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Structural Identification of the Diester Preen-Gland Waxes of the Red Knot (*Calidris canutus*)

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The intact C_{32}−C_{48} diester wax esters of the preen gland of the migrating bird *Calidris canutus* are shown, using synthesized standards, to comprise predominantly C_{12}−C_{16} alkane-1,2-diols esterified with octanoic, decanoic, and dodecanoic acid at one position, and with predominantly even-numbered carbon fatty acids at the other position.

Birds protect their feathers against adverse environmental factors with wax produced in a special gland, the preen gland. This gland is located on the rump at the bottom of the tail feathers in the dorsal caudal tract. The chemical composition of these preen-gland waxes is very complex and varies significantly from order to order.[1] Preen-gland secretions consist predominantly of monoester waxes. Their fatty acid and alcohol moieties may possess straight chain, monomethyl alkyl, polymethyl alkyl, or even more complex carbon skeletons. Usually a mixture of fatty acids and alcohols with varying chain lengths and degree and location of substituents occurs, resulting in a complex mixture composed of hundreds of individual wax esters. Some birds use diester waxes instead of monoesters.[2,3] The physiological function of preen waxes is still a matter of debate. They have been suggested to protect birds against wetting, to make the feather flexible (reducing damage), to play a role as antiparasitic compounds, and to provide UV protection.

The preen-wax composition of captive and free-living *Calidris canutus* L., 1758, subspecies *islandica* (Linnaeus, 1767), belonging to the sandpiper family Scolopacidae, was examined by analysis of intact wax esters with GC−MS and GC−MS/MS.[4] The preen lipids are composed of only of C_{21}−C_{30} monoester waxes composed of C_7−C_{16} 2-methyl and 2,6-, 2,8-, and 2,10-dimethyl fatty acids esterified with C_{11}−C_{22} straight-chain and methyl-branched alcohols (Figure 1a; wax pattern A) in good agreement with hydrolysis studies.[5] However, study of the preen waxes of captive male and female *C. canutus islandica*, which maintain their natural mass and moult cycles, revealed significant changes over the annual cycle in the chemical composition of the preen waxes,[6] as has been observed for ducks.[3] This shows that the molecular composition of preen wax is highly dynamic, in contrast to the general belief that preen waxes can be used in the taxonomic classification of birds (which assumes a constant composition over the annual cycle). In spring, the first change in the preen-wax composition was observed. Although still composed of monoester waxes, vernal preen wax has a total carbon number distribution in the range C_{24}−C_{26} and C_{30}−C_{38}, and the esters were predominantly based on C_{17}−C_{19} alcohols (Figure 1b; wax pattern B). At the end of May, when the birds were ready to depart for the high Arctic, the preen-wax composition changed again drastically and was now predominantly diester wax-based (Figure 1c; wax pattern C). In early summer, the wax pattern starts to switch back to pattern B. These changes co-occur with changes in other physiological parameters such as body mass and plumage.[6]

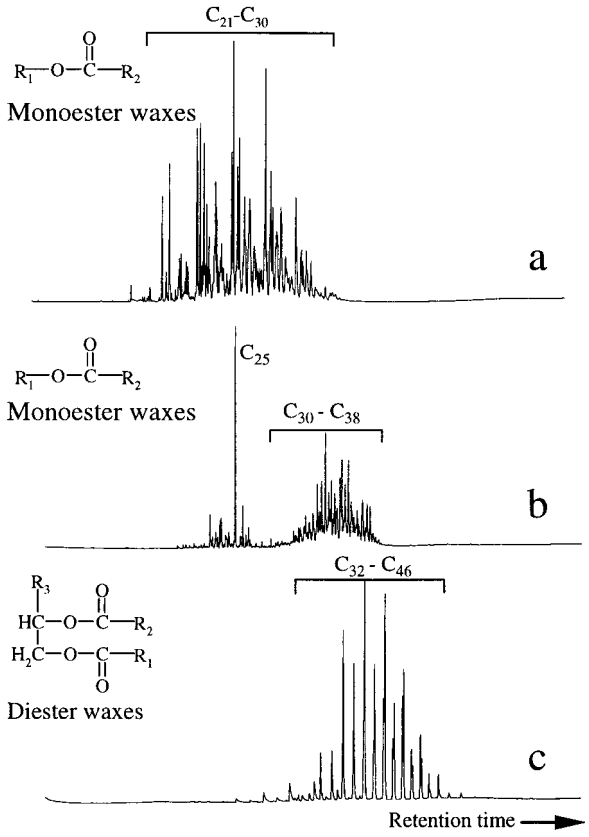

Figure 1. Gas chromatograms of samples of intact preen waxes produced by captive *C. canutus* obtained on (a) January 1998 (wax pattern A), (b) 19 May 1998 (wax pattern B), and (c) 2 June 1998 (wax pattern C), revealing the three different wax patterns observed over the annual cycle. Similar shifts in wax patterns occur in free living birds as revealed by the preen-wax composition of red knots from Delaware Bay just before their departure to the Arctic.[6]

Here we describe in detail the chemical composition of high-molecular-weight diester preen waxes of *C. canutus*. Saponification of the diester wax resulted in the formation

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Table 1. Relative Composition of Fatty Acids and Alkane-1,2-diols Formed upon Hydrolysis of the Diester Preen Waxes of *C. canutus islandica*[a]

fatty acids	rel. amount[b] (mol %)	1,2-alkane-diols	rel. amount[c] (mol %)
C_8	10.8	C_{12}	17.6
C_9	2.6	C_{13}	4.0
C_{10}	8.5	C_{14}	60.1
C_{11}	1.3	C_{15}	1.6
C_{12}	29.9	C_{16}	12.3
C_{13}	5.2	C_{17}	0.6
C_{14}	20.2	C_{18}	3.8
C_{15}	2.5		
C_{16}	15.6		
C_{17}	1.0		
C_{18}	2.5		

[a] Analyzed as their methyl ester and TMS ether derivatives. [b] Determined from integration of the *m/z* 74+87 mass chromatogram. These ions represent the most abundant ions in the mass spectra of the fatty acids. [c] Determined from integration of the *m/z* 73+103+147+243+257+271+285+299+313+327 mass chromatogram. These ions represent the most abundant ions in the mass spectra of the alkanediols.

of $C_{12}-C_{18}$ alkane-1,2-diols and C_8-C_{18} straight-chain fatty acids in a compound class ratio of about 1:2. The alkane-1,2-diols were identified as their TMS derivatives by comparison of their mass spectra with those reported in the literature.[7] Their electron impact (EI) mass spectra are characterized by fragment ions at *m/z* 73, 103, 147, and an abundant M-103. The carbon number distribution of the diols exhibits a strong even-over-odd predominance and is dominated by tetradecane-1,2-diol (Table 1). The fatty acids released also show a strong even-over-odd carbon number predominance, dominated by C_{12}, C_{14}, and C_{16}. However, it is likely that during workup some of the fatty acids (especially C_8) were lost due to their volatility. The results indicated that the high-molecular-weight waxes are diesters composed of $C_{12}-C_{18}$ alkane-1,2-diols and C_8-C_{18} straight-chain fatty acids.

Subsequent analysis of the intact diester wax by GC−MS using EI ionization revealed complex mass spectra (e.g., Figure 2a) in which no molecular ions were encountered. GC−MS with chemical ionization (CI) revealed that the diester waxes are $C_{32}-C_{48}$ components. Several homologous series of diesters were encountered (Figure 3). The major series (series 1) possesses a base peak at *m/z* 127 ($C_7H_{15}CO^+$) and two abundant fragment ions at M-143 ($-C_7H_{15}COO^\bullet$) and M-144 ($-C_7H_{15}COOH$) in their EIMS. These compounds seem to be composed of octanoic acid esterified to one of the hydroxy groups of the alkane-1,2-diol moiety.

In an attempt to fully determine the structure of this series of diesters, two authentic standards, 1,2-tetradecanediyl 1-tetradecanoate 2-octanoate (**1**) and 1,2-tetradecanediyl 1-octanoate 2-tetradecanoate (**2**), were synthesized. Both standards are diesters of tetradecane-1,2-diol esterified to octanoic acid and tetradecanoic acid but differ in the position at which the acids are esterified. The mass spectra of the diester standards **1** and **2** (Figure 2b,c) are virtually identical and do not have a molecular ion. They are both characterized by fragment ions at *m/z* 127 ($C_7H_{15}CO^+$), *m/z* 211 ($C_{13}H_{27}CO^+$), *m/z* 338 (M-228; $-C_{13}H_{27}COOH$), *m/z* 339 (M-227; $-C_{13}H_{27}COO^\bullet$), *m/z* 422 (M-144; $-C_7H_{15}COOH$), and *m/z* 423 (M-143; $-C_7H_{15}COO^\bullet$), all associated with loss of one of the two acid moieties. Apparently, there is no preference for loss from a specific position, because the relative intensities of all these fragments are virtually identical. The fragment ion at *m/z* 194 ($C_{14}H_{26}$) is probably formed by loss of both octanoic acid and tetradecanoic acid and, thus, provides information on the length of the chain of the 1,2-diol moiety. However, a smaller ion at *m/z* 166 ($C_{12}H_{22}$) is also present in both spectra, indicating that this reasoning should be used with some caution. The formation pathway of the fragment ion at *m/z* 267 observed in both spectra is at present enigmatic. The authentic standards coelute with each other and with the C_{36} member of the most abundant series of the diester preen-wax type C (Figure 3; series a) on two capillary columns with different stationary phases (CP Sil-5 and DB-1701). Because both the GC and MS characteristics for the authentic standards are identical, it is not possible to conclude whether **1** or **2** or both are present in the diester preen wax.

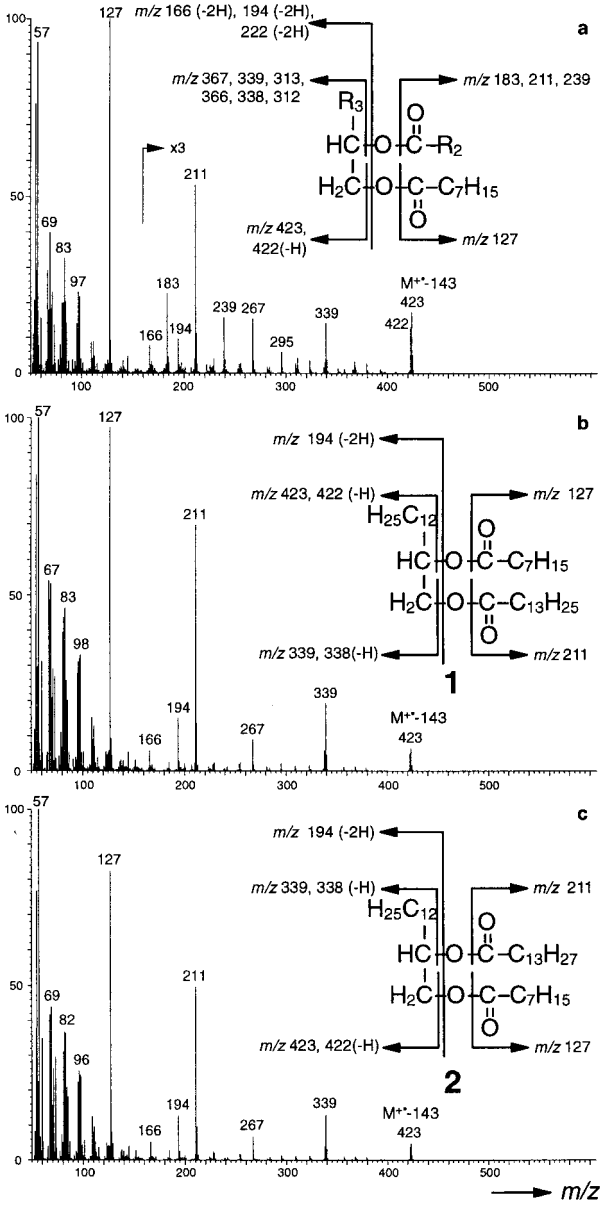

Figure 2. Mass spectra (subtracted for background) of (a) the C_{36} member of series 1 of diester waxes (cf. Figure 3), (b) 1,2-tetradecanediyl 1-tetradecanoate 2-octanoate (**1**), and (c) 1,2-tetradecanediyl 1-octanoate 2-tetradecanoate (**2**). Tentative fragmentation patterns are indicated.

Comparison of the mass spectrum of the C_{36} member of series 1 with those of **1** or **2** (Figure 2) indicates that additional coeluting C_{36} diesters containing an esterified octanoic acid moiety must be present. Additional acyl fragment ions (i.e., *m/z* 183 and 239) are observed in lower abundance (e.g., Figure 2a) and are accompanied by

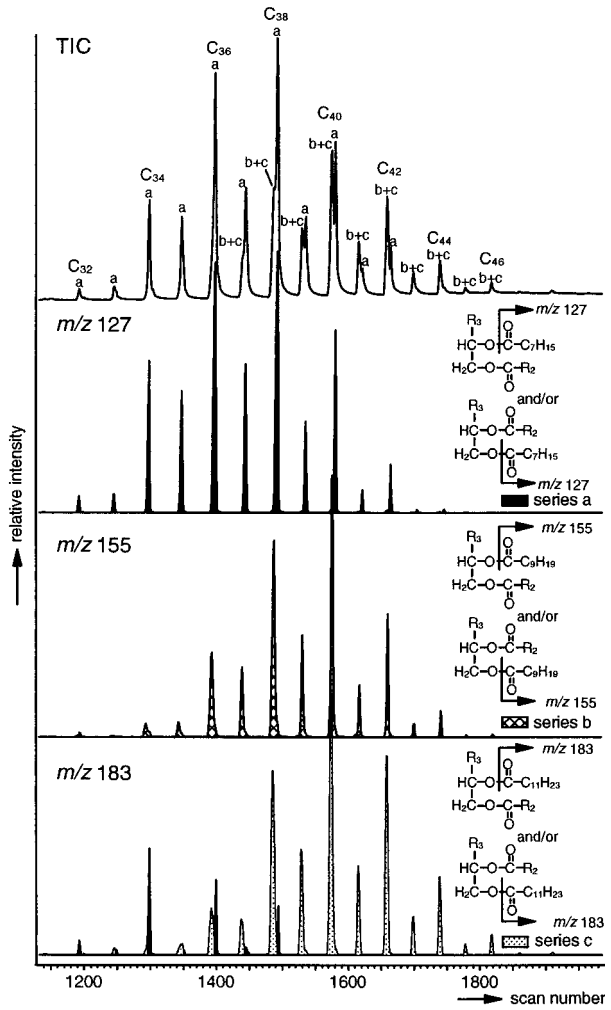

Figure 3. Total ion current (TIC) and mass chromatograms of *m*/*z* 127, 155, and 183 of the red knot preen-wax composition type C (cf. Figure 1c), revealing that it is mainly composed of three "series" of diesters (a, b, and c) characterized by the presence of esterified octanoic, decanoic, and dodecanoic acids, respectively.

corresponding M − $C_xH_{2x+1}COO^•$ and M − $C_xH_{2x+1}COOH$ fragment ions. This suggests that one of the hydroxy groups is esterified with alkanoic acids with variable chain lengths. To keep the molecular weight the same, the chain length of the alkane-1,2-diol is varied in the opposite direction, as is also evident from the ions at *m*/*z* 166 and, to a lesser extent, *m*/*z* 222. Thus, the peaks in the mass chromatogram of *m*/*z* 127 (Figure 3) are composed of various structural isomers, which have in common that they are all esterified at one position with octanoic acid. Members of this homologous series with an even number of carbon atoms are predominantly composed of C_{12}−C_{18} even-numbered carbon 1,2-diols esterified with octanoic acid and an even-numbered carbon fatty acid. The composition of the clusters with an odd number of carbon atoms is more complicated since both odd alkane-1,2-diols and odd fatty acids participate in the various structural isomers present.

The second series of components in the *C. canutus* diester preen wax elutes just before the series with *m*/*z* 127 as the base peak (Figure 3). It is composed of several homologous series that are only partly separated from each other. Two important homologous series are characterized by base peaks at *m*/*z* 155 and 183 (series b and c in Figure 3). In analogy to series 1, they are composed of C_{12}−C_{18} even-numbered carbon 1,2-diols esterified with decanoic and dodecanoic acid, respectively, and with predominantly even-numbered carbon fatty acids at the other position.

This is the first time intact diester preen-wax esters of birds have been unambigiously identified. Our data demonstrate that the "biological clock" of migrating birds, apart from many other physiological parameters,[8] also effects the chemical and, thus, physical characteristics of the preen wax. From a chemical perspective, this change is rather drastic inasmuch as the diester waxes are based only on straight chain components, whereas the monoester waxes are predominantly based on branched components. The reason for the change in preen-wax composition is presently not known. We have suggested that this may be related to the need for a brilliant plumage at the time of mate selection at the Arctic breeding grounds,[6] but this hypothesis needs to be experimentally verified.

Experimental Section

General Experimental Procedures. Details of experimental and analytical methods have been published elsewhere.[4] In brief, preen waxes were harvested with cotton wool by massaging the nipple, and the intact waxes were extracted with ethyl acetate and analyzed by GC and GC−MS. Waxes were saponified by refluxing (1 h) in 1 M KOH−MeOH. The formed fatty acids and alcohols were subsequently derivatized to their corresponding methyl esters and trimethyl silyl ethers, respectively, by treatment with diazomethane and bis(trimethylsilyl)trifluoroacetamide (BSTFA). GC−MS with chemical ionization (with air as the reagent gas) was performed on HP 6890 gas chromatograph coupled to HP 5973 mass spectrometer with a mass range *m*/*z* 50−800 and a cycle time of 0.5 s. Gas chromatographic conditions for these experiments were as described elsewhere.[4]

Preen-Wax Sources. The preen-wax composition of captive red knots, *C. canutus*, belonging to the population wintering in western Europe and breeding in northern Greenland and the Canadian high-arctic islands (subspecies *islandica*[9]) was followed over the annual cycle. In addition, preen waxes of red knots were sampled in May 1998, in Delaware Bay, U.S.A. These birds belonged to the subspecies *rufa* wintering in Tierra del Fuego and breeding in the lower Canadian Arctic.[10] Original feather samples containing preen wax are kept frozen in the collection of The Netherlands Institute for Sea Research (NIOZ).

Synthesis. 1,2-Tetradecanediyl 1-tetradecanoate 2-octanoate (**1**) was synthesized as follows. 2-Hydroxytetradecanoic acid was converted to 2-hydroxytetradecanoic acid octanoate by O-acylation with 1 equivalent octanoyl chloride in the presence of 1 equiv of pyridine (DCM, 0 °C, 3 h) (yield 95%, 96% pure by GC). The resulting acid was reduced with 1 equiv of borane−tetrahydrofuran complex[10] to 1-hydroxytetradecyl 2-octanoate (THF, 0 °C, 12 h). The crude alcohol was converted to diester **1** by O-acylation with 1 equivalent of myristoyl chloride in the presence of 1 equiv of pyridine (DCM, 0 °C, 3 h), and diester **1** was isolated by column chromatography (overall yield 6%, 87% pure by GC): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.09 (1H, m, H-2), 4.22 (1H, dd, J = 3.3, 11.7 Hz, H-1a), 4.04, (1H, dd, J = 6.7, 11.7 Hz, H-1b), 2.30 (4H, t, J = 7.5 Hz, H-2′, H-2″), 0.89 (9H, t, J = 6.8 Hz, H-14, H-16′, H-8″).

1,2-Tetradecandiyl 1-octanoate 2-tetradecanoate (**2**) was synthesized in a similar way. In the first step the conversion to 2-hydroxytetradecanoic acid tetradecanoate was done by O-acylation with 1 equiv of myristoyl chloride (yield 96%, 91% pure by GC). The conversion to diester **2** in the third step was done by O-acylation with 1 equiv of octanoyl chloride (overall yield 10%, 87% pure by GC): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.09 (1H, m, H-2), 4.22 (1H, dd, J = 3.3, 11.8 Hz, H-1a), 4.04, (1H, dd, J = 6.7, 11.7 Hz, H-1b), 2.30 (4H, t, J = 7.5 Hz, H-2′, H-2″), 0.89 (9H, t, J = 6.8 Hz, H-14, H-8′, H-16″); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.6, 173.4 (q, C-1′, C-1″), 71.3 (t, C-2), 64.9 (s, C-1), 34.5, 34.2 (s, C-2′, C-2″), 31.9, 31.7 (s, C-12, C-6′, C-14″), 30.8 (C-3), 29.7, 29.6, 29.5, 29.4, 29.3, 29.1, 29.0, 28.9

(s, C-5−C-11, C-4′, C-5′, C-4″−C-13″), 25.1, 24.9 (s, C-4, C-3′, C-3″), 22.7, 22.6 (s, C-13, C-7′, C-15″), 14.1 (C-14, C-8′, C-16″).

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